Short communication

Anti-Trichomonas vaginalis activity of Hypericum polyanthemum extract obtained by supercritical fluid extraction and isolated compounds

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The anti-Trichomonas vaginalis activity of Hypericum polyanthemum extract obtained by supercritical fluid extraction (50 °C, 150 bar) and the chemical compounds isolated and purified from this extract (benzopyrans HP1, HP2, HP3, and phloroglucinol derivative uliginosin B) were assessed. All samples had anti-T. vaginalis activity; however, HP1 demonstrated the best selectivity against this protozoan (metronidazole-resistant and susceptible isolates), with no cytotoxicity on mammalian cells (selectivity index of 73.97). Moreover, HP1 had activity against a metronidazole-resistant isolate (52% of viable trophozoites) and this effect was higher when tested with a low concentration of metronidazole (23% of viable trophozoites). Experiments demonstrated that all isolated compounds caused damage to the parasites’ membrane (>90% of LDH release) and do not present a notable hemolytic effect, although HP2 and uliginosin B exhibited cytotoxicity against mammalian cells. Therefore, the analyzed molecules are promising prototypes for new antiprotozoal drugs, especially HP1, which seems to improve metronidazole’s effect on a resistant T. vaginalis isolate.

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1. Introduction

Trichomonas vaginalis is a mucosal pathogen which affects the human urogenital tract causing trichomoniasis [1], the most common non-viral sexually transmitted disease. According to the WHO (2001), there are about 170 million cases of trichomoniasis worldwide every year [2]. Studies have indicated that complications related to this disease include infertility [3], pelvic inflammatory disease [4], predisposition to cervical cancer [5], increased prevalence of high-risk human papillomavirus (HPV) infection [6], birth outcomes and increased transmission and acquisition of the human immunodeficiency virus (HIV) [7].

Metronidazole and tinidazole are the only drugs recommended by the FDA for the treatment of T. vaginalis infection; however, resistant isolates have been described [8]. Drug failures appeared as soon as metronidazole was approved for the treatment of trichomoniasis and an alarming increase in infections caused by resistant isolates has occurred [9,10]. In many cases resistance is overcome with prolonged therapy and higher doses of metronidazole, but occasionally patients continue infected [11]. Moreover, high doses are often not well tolerated by patients, with the development of unpleasant side effects including nausea, headache, dizziness, dry mouth and a metallic taste sensation [12].

In the context of improving the therapy of T. vaginalis infection, natural products could be a source of new drugs with high activity and low toxicity, which can be further optimized by synthetic procedures. Plants of the genus Hypericum (Guttiferae) are known for their use in traditional medicine. The most important species, Hypericum perforatum L., has shown several activities, including antimalarial, which was attributed to a major lipophilic constituent, hyperforin, a phloroglucinol derivative [13–15].

Hypericum polyanthemum, a Brazilian native species, has been found to contain in its lipophilic extract uliginosin B, a phloroglucinol derivative, and three benzopyrans: 6-isobutryl-5,7-dimethoxy-2,2-dimethylbenzopyran (HP1), 7-hydroxy-6-isobutyryl-5-methoxy-2,2-dimethylbenzopyran (HP2) and 5-hydroxy-6-isobutyryl-7-methoxy-2,2-dimethylbenzopyran (HP3) (Fig. 1) [16,17]. With the intention of optimizing the method of extraction of these bioactive compounds, a previous study was conducted using supercritical fluid extraction (SFE) technology [18].

Considering the increase in drug resistance and the fact that lipophilic compounds such as benzopyrans and phloroglucinol derivatives have potential antiprotozoal activity, the aim of this study was to investigate the anti-T. vaginalis activity in a fraction of H. polyanthemum...
extract obtained by SFE and isolated compounds: HP1, HP2, HP3, and uliginosin B.

2. Materials and methods

2.1. Plant material, supercritical fluid extraction and quantification of metabolites

The aerial parts of Hypericum polyanthemum Klotzsch ex Reichardt were collected in October 2008, in Caçapava do Sul, Rio Grande do Sul, Brazil, authorized by Conselho de Gestão do Patrimônio Genético (CGEN) and Instituto Brasileiro do Meio Ambiente (IBAMA) 003/2008 P 02000.001717/2008-60. Authentication of the plant species was established by Dr. Sérgio Bordignon (UNILASALLE-RS-Brazil) and the voucher specimen deposited in the herbarium of the Universidade Federal do Rio Grande do Sul (UFRGS) (ICN) (Bordignon 1405).

The dried and powdered plant material (180 g) was successively extracted with supercritical carbon dioxide under pressures of 90, 120, 150 and 200 bar at different temperatures (40, 50 and 60 °C). The compounds were quantified in each fraction by HPLC [19,20], and the optimum temperature for extraction of bioactive compounds by SFE was 50 °C. The sample chosen for biological assays (50 °C, 150 bar) was analyzed and the quantification of HP1, HP2, HP3, and uliginosin B reached values of 141.3, 117.5, 102.6 and 352.5 mg per g/extract, respectively [18].

2.2. Isolation of bioactive compounds

The compounds were isolated from SFE fractions by column chromatography on silica gel 60 using mixtures of n-hexane: dichloromethane of increasing polarity as eluent, and further purified by preparative thin-layer chromatography (TLC) on 20 cm×20 cm glass supported plates covered with 0.5 mm silica gel GFls54 (Merck) and n-hexane: dichloromethane (1:1) as solvent. The bands were detected under UV light (254 nm), and the purity (≥90%) was confirmed by HPLC analysis, taking into account the retention time of the compounds compared with standards (authentic samples) isolated from H. polyanthemum, as described by Ferraz et al. [16,21], where the identities and purities of the compounds were confirmed by NMR spectroscopic analysis.

2.3. Culture of Trichomonas vaginalis

The T. vaginalis isolate sensitive to metronidazole used in this study, 30236 (ATCC), was kindly donated by Prof. Dr. Geraldo A. De Carli (PUCRS, Brazil), and the metronidazole-resistant isolate, TV-LACM2 (a fresh clinical isolate) was obtained from a female patient from the Laboratório de Análises Clínicas e Toxicológicas, Faculdade de Farmácia, UFRGS, Brazil (protocol number 18923 approved by the UFRGS Ethic Committee). Trichomonads were cultured axenically in vitro in trypicase-yeast extract-maltose (TYM) medium (pH 6.0), supplemented with 10% heat-inactivated bovine serum, and incubated at 37 °C [22]. Organisms in the logarithmic phase of growth and exhibiting more than 95% viability and normal morphology, were harvested, centrifuged, washed three times with phosphate-buffered saline (PBS) and resuspended in fresh TYM medium for cytotoxicity assays.

2.4. Anti-Trichomonas vaginalis assay

The cytotoxicity of the SFE extract and compounds against T. vaginalis was determined in vitro. The extract at concentration of 5.2 mg/mL and the isolated compounds at 1.0 mg/mL were diluted with sterile water using DMSO (0.62%) as vehicle of solubilization and a final eight-fold dilution for all samples tested was obtained. A fluorimetric method based on the resazurin salt reduction proportional to the viable trophozoites was used for testing the SFE extract activity [23]. The resazurin method was not employed when the isolated compounds were tested because of their natural fluorescence, which interferes with resazurin fluorescence. To perform the quantitative assay with the isolated compounds, HP1, HP2, HP3, and uliginosin B were incubated at 37 °C for 24 h at final concentrations of 250, 125 and 62.5 μg/mL with 5.0×10^5 trophozoites/mL. The T. vaginalis viability was determined by counting using a hemocytometer. Three controls were conducted: a negative control only with trophozoites; control of DMSO used as vehicle, and a positive control: 8.0 μM metronidazole (MTZ) (total impurities: ≤0.0005% phosphorus and ≤0.1% insoluble matter; Sigma-Aldrich, St. Louis, MO, USA) for the metronidazole-sensitive T. vaginalis isolate (30236), and 289 μM MTZ for the metronidazole-resistant isolate (TV-LACM2) [23]. The results were obtained from at least three independent experiments, in triplicate, and expressed as the percentage of living parasites after 24 h of incubation considering motility and normal morphology (percentage of living organisms compared to negative control). The concentration required to inhibit 50% of the parasites grow (IC_{50}) was determined by linear regression.

2.5. In vitro metronidazole susceptibility assay

The metronidazole susceptibility assay was carried out in accordance to Tachezy et al. [24]. Briefly, the assay was performed in 96-well microtiter plates, where metronidazole (Sigma-Aldrich, USA) at 74,000 μM was added in the first well and an eight-fold dilution was obtained. Next, the trophozoites (metronidazole-resistant isolate) were added with trophozoites (metronidazole-resistant isolate) were added (5.0×10^5 trophozoites/mL) and incubated for 24 h at 37 °C. In addition, different concentrations of HP1 (62.5, 125 and 250 μg/mL) were tested with 8.0 μM of metronidazole. The T. vaginalis viability was determined as described in the anti-Trichomonas vaginalis assay.

2.6. Lactate dehydrogenase assay

LDH release was analyzed in relation to the toxicity of the all isolated compounds towards parasites. Compounds at a final concentration of
125 μg/mL were incubated with metronidazole-sensitive *T. vaginalis* isolate (5.0 × 10⁴ trophozoites/mL) and 1.0 mM protease inhibitor cocktail (Sigma) at 37 °C, for 24 h. In addition, HP1 was incubated with metronidazole-resistant *T. vaginalis* isolate (5.0 × 10⁴ trophozoites/mL). The LDH released by both parasites was measured spectrophotometrically in the supernatant, using a commercial kit (Labtest). Parasites untreated and lysed with liquid nitrogen for 1 min were used as negative and positive controls, respectively. The LDH released was calculated as a percentage of the positive control, considered 100% of cellular damage.

2.7. Hemolytic assay

The hemolytic assay was performed according to Gauthier et al. [25] with some modifications. Type O positive blood from healthy human volunteers was collected with Alsever’s solution (1:1) and centrifuged at 2000 rpm for 5 min. The erythrocyte fraction was washed three times with PBS (pH 7.0) and resuspended to give a 1.0% suspension. Compounds under investigation were dissolved using DMSO and purified water to achieve a final concentration of 125 μg/mL. Using microtubes, the 1.0% erythrocyte suspension was mixed with the samples solution and/or water to obtain a final concentration of approximately 0.7% of erythrocytes. The microtubes were incubated at 37 °C under horizontal agitation for 1 h and then centrifuged at 3000 rpm for 5 min. The absorbance of the supernatant was measured at 540 nm. The experiment was performed in triplicate, and the percentage of hemolysis was calculated in comparison to 100% hemolysis attributed to the hemolytic action of a saponin commercially available (QuilA®—Superfos, Denmark) [26].

2.8. Cytotoxicity against the VERO cell line

Cell viability was determined by a colorimetric method [27], using VERO cells (African Green Monkey Kidney, ATCC CCL-81) and MTT reagent (Sigma-Aldrich, USA). Briefly, VERO cells were cultured in Eagle’s

![Fig. 2. Effect of different concentrations *H. polyanthemum* compounds against *T. vaginalis* isolate sensitive to metronidazole (ATCC-30236). The positive control was 8.0 μM of metronidazole (MTZ). Data represent means ± standard deviation of at least three experiments. Different letters (a,b,c,d) at the same concentration indicate significant differences among the compounds (P<0.05%).](image-url)
minimal essential medium supplemented with 10% fetal bovine serum and antibiotics (penicillin 100 IU/mL; streptomycin 100 μg/mL). Experiments were performed in 96-well microtiter plates, where a suspension of 4.0 × 10^{4} cells/mL per well was incubated in a humidified atmosphere with 5% CO_{2} at 37 °C. When cells reached more than 80% confluence, the medium was replaced and the cells treated with \textit{H. polyanthemum} extract at concentrations of 0.65, 0.325, 0.162, and 0.081 mg/mL or with the isolated compounds at final concentrations of 250, 125, 62.50 and 31.25 μg/mL, dissolved in DMSO (0.62%). After 24 h of incubation, stock MTT solution (2.0 mg/mL) was added and plates were incubated for a further 3 h. DMSO was added and the plates incubated for 10 min to stop the reaction and to dissolve the insoluble purple formazan. The amount of MTT-formazan present is directly proportional to the number of living cells and was determined by measuring the optical density (OD) at 550 nm. Three wells per dose were analyzed in three different experiments, and the results were expressed as the percentage of viable cells in comparison to negative control (untreated cells). The concentration of the compound that killed 50% of the cells (CC50) was calculated by linear regression.

2.9. Statistical analysis

The data were subjected to one-way analysis of variance (ANOVA) using a probability value of \( P < 0.05\). Tukey's test was used to identify significant differences between means among the different treatments (SPSS Software).

3. Results and discussion

Taking into account that approximately 5% of all clinical cases of trichomonosis are caused by \textit{T. vaginalis} isolates with some level of resistance to metronidazole, the treatment constitutes a major therapeutic challenge and the options are limited [12,28]. This study demonstrated the anti-\textit{T. vaginalis} activity of \textit{H. polyanthemum} SFE extract (no viable trophozoites at 325 μg/mL) (data not shown) and isolated compounds (from 62.5 to 250 μg/mL).

The antiprotozoal activity of all isolated compounds was dose-dependent, being the phloroglucinol derivative the most active (less than 20% of trophozoites viable) (Fig. 2), presenting an IC_{50} value of 121.96 μM (Table 1). The benzopyran HP1 reduced the number of viable trophozoites by around 70% (Fig. 2), and among the benzopyrans it presented the lowest IC_{50} value (226.50 μM) (Table 1). HP2 exhibited less antiprotozoal activity than HP1 and also presented marked toxicity against VERO cell line (Fig. 3). On the other hand, HP3 was not found to be toxic, but its antiprotozoal activity was unremarkable (60% of viable trophozoites), corresponding to half the activity of HP1 (Fig. 2).

The hemolytic and LDH assays were performed in order to examine the action of isolated compounds on membranes, indicating selectivity for the parasites or human cells. In the hemolysis test, none of samples
caused any notable hemolytic effect. However, the LDH assay showed that all compounds caused damage to the parasite membranes, including HP1 toward membranes of T. vaginalis metronidazole-resistant isolate (>90% of LDH release) (Fig. 4).

The SFE extract and the isolated compounds were evaluated for their toxicity against mammalian cells using the VERO cell line. The compounds presented a CC50 ranging from 213.91 to 16,753.33 μM (Table 1); being HP2 as the most toxic (Fig. 3). On the other hand, considering their molecular weights, uliginosin B presented a higher cytotoxic effect against VERO cells, represented by the lowest CC50 value. Nevertheless, the H. polyanthemum extract was not toxic to mammalian cells (more than 83% of viable cells), even though it contained 35.2% of uliginosin B [18].

The selectivity index (SI) of the compounds defined as the ratio of cytotoxicity to biological activity (SI = CC50 VERO cells/IC50 parasites) (Table 1) were calculated. It is generally considered that biological efficacy is not due to in vitro cytotoxicity when SI ≥ 10 [29]. Thus, a low selectivity index is indicative of the high in vitro cytotoxicity of the sample and the lack of selectivity for the parasites. Uliginosin B, a molecule that appeared very promising when evaluated only for antiprotozoal purposes, presented a low SI value (1.75), thereby showing itself not to be selective. By contrast, the benzopyran HP1 had a SI value of 73.97, proving it to be the most promising compound with significant antiprotozoal activity and selectivity. Therefore, benzopyrans and the phloroglucinol derivative are molecules with selectivity for the trichomonads, and the mechanism of cytotoxicity may involve loss of membrane integrity (high LDH values).

Furthermore, HP1 presented activity against a metronidazole-resistant clinical isolate (TV-LACM2), being able to kill 47% of trophozoites at the highest concentration (250 μg/mL) (Fig. 5). The metronidazole MIC for this isolate was measured and, different to ATCC-30236, with a low MIC value (8.0 μM), the TV-LACM2 isolate presented a MTZ MIC value of 289 μM. In addition, HP1 was evaluated with 8.0 μM of metronidazole and the effect was improved considerably, indicating a successful association (Fig. 5). It could be suggested that the HP1 antiprotozoal activity against the resistant isolate was due to an interaction with the membrane lipids of the parasite (100% of LDH release), resulting in cell damage and, consequently, cell death. Lipophilicity is an important consideration in the design of novel antiparasitic drugs, since long alkyl chains can interact with membrane lipids allowing the penetration of the compounds into the cytoplasm of the parasite [30,31]. The results indicate that HP1 and metronidazole act by different mechanisms of action; the latter is activated in the hydrogenosomes of the parasites, resulting in the production of nitrogen radicals that damage DNA [12]. Consequently, an existing resistance to metronidazole would not influence the susceptibility to HP1. However, the exact mechanism of the cytotoxic effect of HP1 remains to be elucidated.

Comparison of these results with those of other studies is particularly complex because different parasites, substances and methods were used to measure activity. Notwithstanding, other authors have found that hyperforin, a phloroglucinol derivative, and 4-methoxy-2,2-dimethyl-6-(2-(2,4-dihydroxy)phenyl-transethenyl) chromene, which possesses a benzopyran nucleus, demonstrated activity against Plasmodium falciparum, with IC50 values of 1.5 μM and 30.1 μM, respectively [13,32]. Uliginosin B presented an IC50 value 81-fold higher than that of hyperforin, and the IC50 value of HP1 was 7.5-fold higher than the substance which possesses a benzopyran nucleus. This difference could be due to the molecular feature of uliginosin B, a dimeric phloroglucinol derivative which differs from that of hyperforin, a phloroglucinol presenting a bicyclonane skeleton substituted with several isoprene chains. On the other hand, the similar antimalarial activity observed with the benzopyran compounds could be attributed to the close related structures. Nevertheless, it is important to underline the fact that this is the first report regarding the anti-T. vaginalis activity of both Brazilian Hypericum extracts and isolated compounds. Although the compounds investigated did not exhibit pronounced activity against T. vaginalis, they show promise as prototypes for new antiprotozoal drugs, especially HP1, which improved the action of metronidazole against a resistant T. vaginalis isolate. Further studies on structural modifications to these compounds are being conducted by our group in order to enhance the in vitro antiprotozoal activity.

Conflict of Interest statement

The authors have declared that there is no conflict of interest.

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